Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Chemical Biology

# Bacterial enzymes for lignin depolymerisation: new biocatalysts for generation of renewable chemicals from biomass

Timothy D. H. Bugg, James J. Williamson and Goran M. M. Rashid

**Abstract**

The conversion of polymeric lignin from plant biomass into renewable chemicals is an important unsolved problem in the biorefinery concept. This article summarises recent developments in the discovery of bacterial enzymes for lignin degradation, our current understanding of their molecular mechanism of action, and their use to convert lignin or lignocellulose into aromatic chemicals. The review also discusses the recent developments in screening of metagenomic libraries for new biocatalysts, and the use of protein engineering to enhance lignin degradation activity.

**Addresses**

Department of Chemistry, University of Warwick, Coventry, CV4 7AL, United Kingdom

Corresponding author: Bugg, Timothy D.H (T.D.Bugg@warwick.ac.uk)

**Current Opinion in Chemical Biology** 2020, **55**:26–33

This reviews comes from a themed issue on **Biocatalysis and biotransformation**

Edited by **Jason Micklefield and Dominic J. Campopiano**

For a complete overview see the Issue and the Editorial

https://doi.org/10.1016/j.cbpa.2019.11.007



**Keywords**

Lignin depolymerisation, Dye-decolourising peroxidases, Multi-copper oxidases, Beta-etherases, Biosensor.

## Introduction

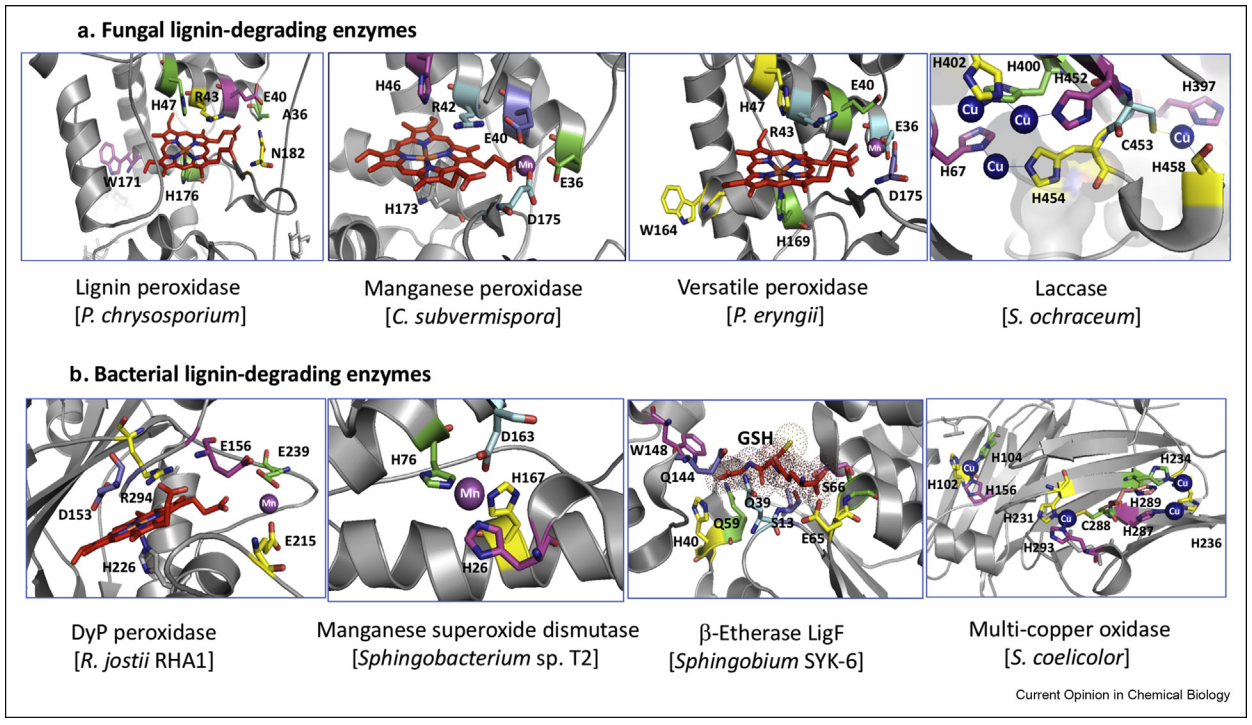
Lignin is an aromatic heteropolymer comprising 15–30% of the lignocellulose cell wall of plant biomass, and it is the most abundant source of renewable aromatic carbon in the biosphere. Given the need to reduce greenhouse gas emissions in the 21st-century society, there is considerable academic and commercial interest in finding new sustainable biocatalytic routes to fuels and chemicals from renewable sources of carbon such as plant biomass [1]. For aromatic chemicals, lignin is an obvious starting point, but it is a very challenging polymer to deconstruct, owing to the presence of non-hydrolysable ether C–O and C–C bond linkages, poor solubility in aqueous solution, and other technical challenges [2].

The search for microbial enzymes to deconstruct lignin has until recently focussed on white-rot basidiomycete fungi, such as *Phanerochaete chrysosporium*, that produce extracellular lignin peroxidases, manganese peroxidases, and multi-copper laccases that can attack lignin (see Figure 1) [3]. However, these fungal enzymes are often challenging to express in high yield, and their fungal hosts are not readily amenable to genetic modification for metabolic engineering. Hence, since 2010, there has been a resurgence of interest in lignin-oxidising enzymes from soil bacteria. A number of soil bacteria have been identified that can depolymerise lignin, mainly in the actinobacteria and α- and γ-proteobacteria phyla, albeit less rapidly than the most active basidiomycete fungi [4,5]. This article will describe recent developments in the enzymology of bacterial lignin-degrading enzymes, our current understanding of how they attack lignin, and applications for biotransformation.

## Identification and characterisation of bacterial enzymes for lignin depolymerisation

The first bacterial lignin-oxidising enzyme to be identified was peroxidase DypB from *Rhodococcus jostii* RHA1, a member of the dye-decolourising peroxidases, found in bacteria and fungi [6]. Dyp-type peroxidases have activity for dye decolourisation and also oxidation of a range of phenolic substrates [7]. There are four subclasses, A–D, based on sequence alignment, of which classes A–C are found in bacteria, and class D is found in fungi [7]. Although many Dyp-type peroxidases have been identified, only some have been demonstrated to have activity for oxidation of polymeric lignin. Lignin-oxidising Dyp peroxidase enzymes have been identified in *Amycolatopsis* sp. 75iv2 (DypC) [8], *Pseudomonas fluorescens* Pf-5 [9], and *Thermobifida fusca* [10]. Pfl Dyp1B is able to release an oxidised lignin dimer from wheat straw lignocellulose in the presence of $Mn^{2+}$ [9]. The ability to oxidise polymeric lignin *in vitro* correlates in

**Figure 1**




Active sites of lignin-degrading enzymes with important residues for catalysis. a. Fungal lignin-degrading enzymes: Lignin Peroxidase [1LLP] from *Phanerochaete chrysosporium* (W171, H47, R43, H176, E40, A36 and N182); Manganese Peroxidase [4CZO] from *Ceriporiopsis subvermispora* (H46, R42, H173, E40, E36, D175); Versatile Peroxidase [2BOQ] from *Pleurotus eryngii* (W164, H47, R43, H169, E40, E36, D175); laccase [3T6V] from *Steccherinum ochraceum* (H67, H402, H400, H452, C453, H454, H458, H397). b. Bacterial lignin-degrading enzymes; Dyp peroxidase [4HOV] from *Rhodococcus jostii* RHA1 (D153, R294, H226, E156, E239 and E215); Manganese Superoxide dismutase (SpMnSOD1) from *Sphingobacterium* sp. T2 [6GSB] (H26, H76, D163 and H167); β-Etherase LigF [4XT0] from *Sphingobium* sp. strain SYK-6 Glutathione (GSH) binding site (GSH, W148, Q144, H40, Q59, Q39, S13, E65 and S66); Multi-Copper Oxidase [4GXF] from *Streptomyces coelicolor* (H102, H104, H156, H231, H293, C288, H289, H287, H236 and H234). Graphics drawn using PyMol software.

most cases with an ability to oxidise $Mn^{2+}$ by some B-type and C-type Dyp peroxidases [6,8,9], although some A-type Dyps can oxidise lignin model compounds [10].

One of the best-studied hosts for bacterial lignin degradation is *Pseudomonas putida*, a well characterised aromatic degrader that has been verified via different experimental approaches to break down lignin [4,11], and this has been used as a host for metabolic engineering of lignin bioconversion [12]. Lin et al. have recently published a genomic and proteomic analysis of *P. putida* A514, implicating the role of two Dyp-type peroxidase enzymes in lignin breakdown [13]. They report that neither of these Dyp enzymes have activity for $Mn^{2+}$ oxidation [13]. A Dyp-type peroxidase has also been reported from *Pseudomonas* sp. Q18 that is able to degrade alkali lignin [14].

In the actinobacteria, Dyp-type peroxidase from *Saccharomonospora viridis* DSM 43017 has been reported that is active for dye decolourisation at neutral-alkaline pH, unlike most such peroxidases whose optimum pH is typically 3–4, and has been applied to bleaching of eucalyptus kraft pulp [15]. A 38 kDa DyP-type peroxidase has also been reported from *Rhodococcus* sp. that has been applied to kraft pulp bleaching [16]. Detailed mechanistic studies have been reported for a B-type Dyp peroxidase from *Enterobacter lignolyticus* [16]. A kinetic isotope effect of 2.44 was observed using $D_2O_2$ indicating that cleavage of the O–H bond during the formation of the compound I iron-oxo intermediate is rate-limiting, and an inverse solvent kinetic isotope effect was interpreted as evidence for a kinetically significant conformational change [17].

The second class of bacterial lignin-degrading enzymes are multi-copper oxidase enzymes, or laccases, which are best characterised from fungal sources, but have also been found in bacteria. Bacterial multi-copper oxidase from *Streptomyces coelicolor* A3 has been implicated in lignin breakdown, from gene deletion studies in which decreases in acid-precipitable lignin (APPL) were observed [18]. Overexpression of a related SLAC enzyme in *Amycolatopsis* sp. 75iv3 has been shown to lead to 6-fold increases in APPL production, with enhanced syringyl (S) content in the APPL structure, and the

release of monocyclic aromatic products such as vanillin, 4-hydroxybenzoic acid, and 1,4-dihydroxy-3,5-dimethoxybenzene (via aryl-Cα cleavage) [19]. A blue multi-copper oxidase CueO from a lignin-degrading *Ochrobactrum* sp. strain has been characterised kinetically, and its crystal structure determined, showing slight differences in the type I copper centre, compared with fungal laccases [20]. The *Ochrobactrum* CueO was found to catalyse oxidative dimerization of lignin model compounds, but depolymerised lignosulfonate, generating vanillic acid as a product [20]. Multi-copper oxidase CopA enzymes from *P. putida* and *P. fluorescens* Pf-5 have been found to be pseudo-laccases, requiring addition of exogenous $Cu^{2+}$ for activity, and show stoichiometry of 8–10 mol Cu/mol for the holoenzyme, but show similar catalytic properties to other bacterial multi-copper oxidases [21]. Multi-copper oxidases CueO and CopA are also linked to bacterial copper utilisation, so it is likely that they have multiple cellular roles [20,21]. Furthermore, multi-copper oxidase complex Mnx has been demonstrated to oxidise Mn(II) to Mn(IV), and the catalytic mechanism for this process elucidated [22]. A multi-copper oxidase enzyme from *Paenibacillus glucanolyticus* SLM1 has also been kinetically characterised against phenolic substrates [23].

A novel lignin-oxidising manganese superoxide dismutase enzyme has been identified in lignin-degrading *Sphingobacterium* sp. T2 [24], which is able to solubilise organosolv and kraft lignin, and generate a mixture of polymeric and monocyclic aromatic products [24]. Recent studies on this enzyme have shown that the enzyme is able to generate hydroxyl radical via one-electron reduction of hydrogen peroxide, a reaction not normally observed in superoxide dismutase enzymes. The predominant reaction with polymeric lignin is demethylation of the methoxy aryl-substituents, and two amino acid replacements close to the Mn(II) centre were shown to be essential for this reactivity, causing increased solvent access to the Mn(II) centre [25].

The fourth group of lignin-degrading bacterial enzymes is the family of glutathione-dependent β-etherase enzymes, which catalyse the reductive cleavage of the β-aryl ether linkage via attack of glutathione at the β position of an oxidised aryl unit containing an α ketone group. These enzymes were first identified in *Sphingobium* SYK-6, a bacterium with the ability to degrade a range of lignin dimers that are likely to be lignin oxidation products, where dehydrogenases LigD and LigL catalyse oxidation of the benzylic α-hydroxyl group, LigE, LigF are stereospecific β-etherase enzymes for ether cleavage, and LigG catalyses reductive elimination of glutathione to generate a benzylic ketone product [26–28]. Crystal structures of *Sphingobium* SYK-6 LigD, LigO, LigL, LigG have been solved, providing insight into mechanisms of catalysis by these enzymes [29]. Analogous dehydrogenases LigO and LigN and β-etherase LigP have been identified in *Novosphingobium*, and have been shown to catalyse stereospecific β-ether cleavage [30]. A Nu-class glutathione S-transferase was identified in *Novosphingobium* as a β-etherase enzyme [30], which has been shown to act as a glutathione lyase, which can cleave both enantiomers of the intermediate glutathione adduct [31]. A β-etherase enzyme has also recently been identified in the white-rot fungus *Dichomitus squalens* [32].

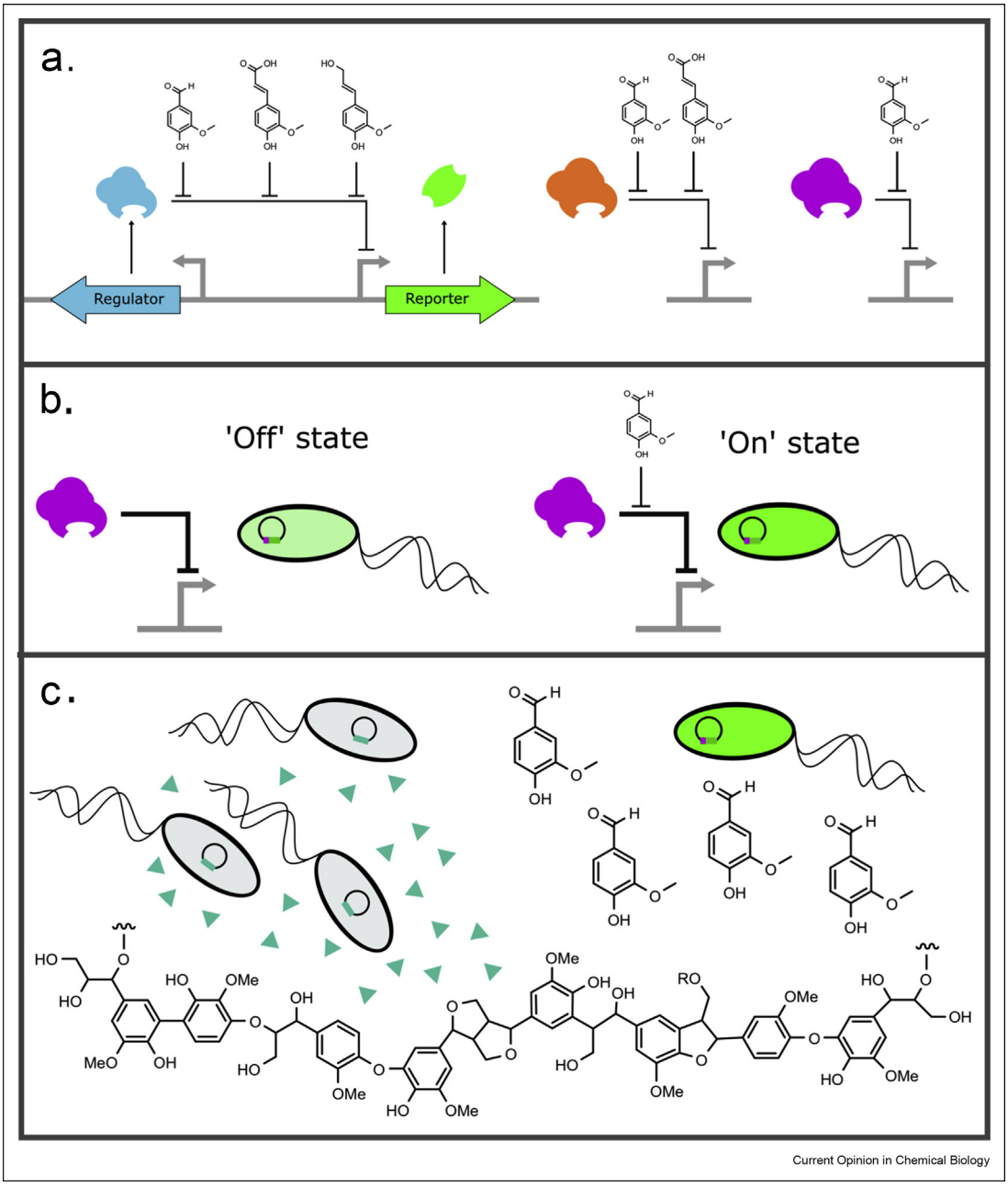
Screening microbes isolated from a range of environments could yield novel enzymes for the degradation of lignin; however, it is accepted that most environmental microbes are unculturable, and the methods used to isolate and characterise those that can be grown are time consuming and work intensive. Hence there is current interest in the use of biosensors for high-throughput screening of metagenomic DNA libraries, as shown in Figure 2. A biosensor comprises of a genetic regulatory unit, which is activated by a specific compound, coupled to a reporter gene, which gives a measurable output such as fluorescence or luminescence. Several biosensors have been developed for a range of compounds associated with the breakdown of lignin, including vanillin [33,34], protocatechuate [35], and phenylpropenoic acids such as ferulic and p-coumaric acid [36]. These regulatory units have been discovered by screening regulator-reporter gene fusion libraries [33] or using known genetic regulation towards a target compound [36]. Rounds of mutagenesis and selection can be used to tighten the regulation towards a specific compound or improve the range of expression in the 'on' and 'off' states [35].

Genomic DNA is typically screened in ~40 kb fosmids in *E. coli*, incubated with lignin or lignin-like substrates, then the reporter strain containing the biosensor is added, and the output measurement taken to assess degradation of the substrate. Positive clones are then investigated to discover the genes responsible for activity. The success rate of these strategies demonstrates their worth compared to traditional methods of screening. Using the vanillin and syringaldehyde sensor strain, Ho et al. screened 42,520 clones and had 147 positive clones [33]. Metagenomic analysis from lignin-treated sugarcane soil has also recently identified unculturable microbial sequences with lignin-degrading activity [37].

## Biotransformation of lignin by lignin-depolymerising enzymes

Our understanding of exactly how lignin-degrading enzymes attack polymeric lignin is still very incomplete. As shown in Figure 3, for the β-aryl ether structure which is the major structural unit found in polymeric lignin, there are a number of possible sites for oxidation or oxidative cleavage. In the relatively few cases where the

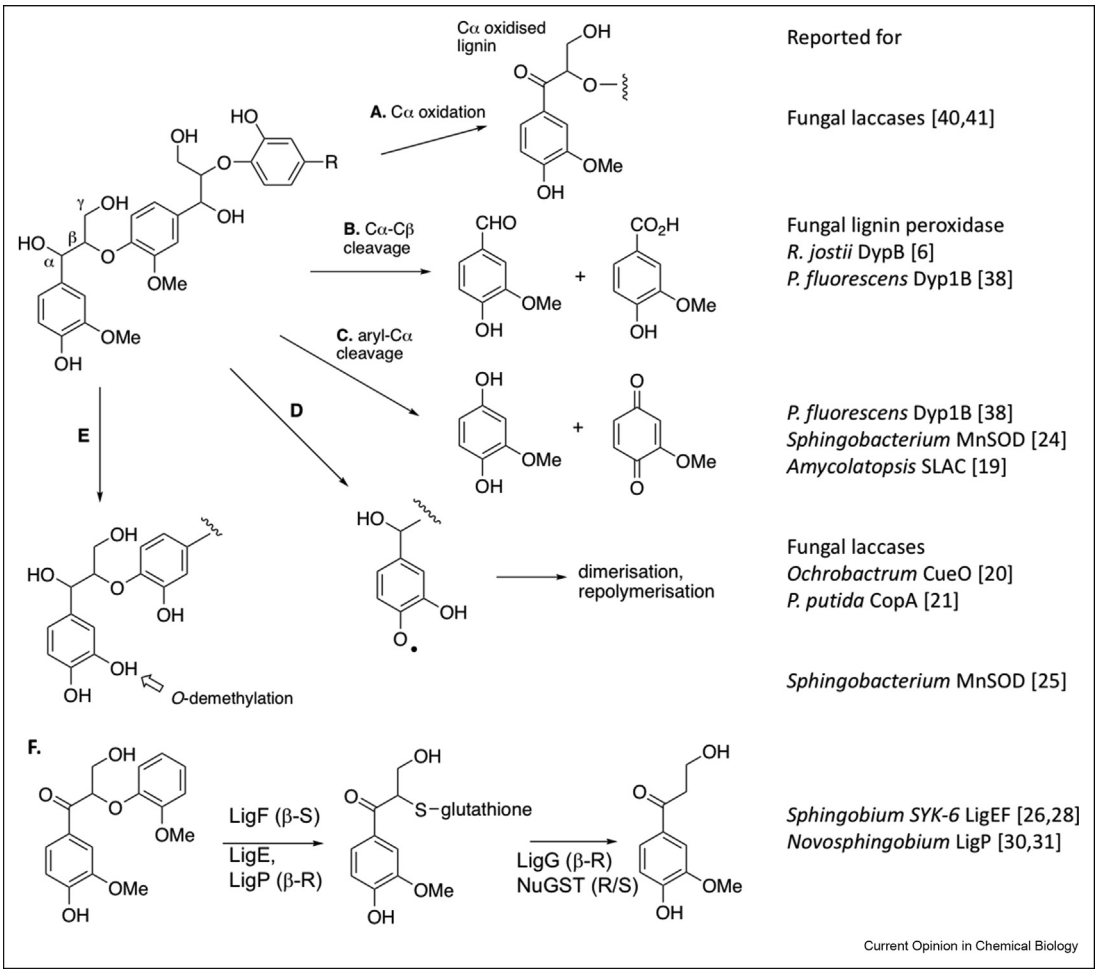
**Figure 2**




Biosensors for lignin-degrading enzyme discovery. a. A biosensor comprises of a genetic regulatory unit, which represses the reporter gene promoter, and this repression is de-repressed by binding of the inducer molecule. Different gene regulators may have different specificity, and their specificity can be altered via protein engineering. b. This in turn leads to expression of the reporter gene, which gives a measurable output such as fluorescence or luminescence. c. Strains containing a library of environmental DNA are then incubated with lignin or lignin like substrates, and then the reporter strain is added and the reporter output is measured.

site of reaction has been studied, studies may be based on the use of lignin dimer model compounds, or the site of reaction implied by structure of low molecular weight product released. A further complication is that products obtained are dependent on the type of lignin used: a study of the biocatalytic and chemocatalytic conversion of a set of lignins revealed different products from different lignin preparations, but showed that lignins containing high β-O-4 content released higher yield and number of products [38], providing some evidence that this linkage is probably the major site of attack in polymeric lignin.

**Figure 3**

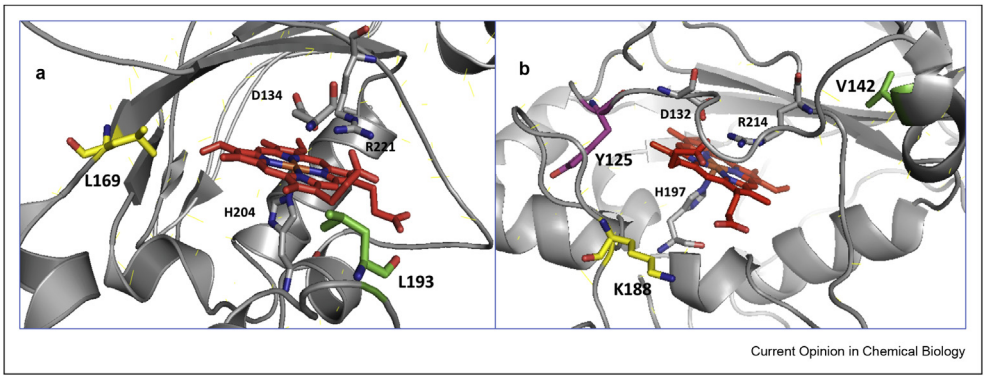



Different enzyme-catalysed reactions of β-O-4 guaiacyl (G) unit found in softwood lignin, showing different types of product, and enzymes reported to carry out that reaction.

For the bacterial Dyp-type peroxidases, the detection of vanillin from a lignin dimer substrate using *R. jostii* DypB indicated that Cα-Cβ oxidative cleavage (route B, Figure 3) had occurred, although dimerization via coupling of phenoxy radicals also occurred [6]. *P. fluorescens* Dyp1B had been shown to release products from Cα-Cβ cleavage (route B) or aryl-Cα cleavage (route C, Figure 3) from polymeric lignin substrates [38], and can release a lignin dimer product containing an oxidised ketodiol sidechain from treatment of wheat straw lignocellulose [9]. For *Sphingobacterium* MnSOD, the major site of reaction appears to be demethylation (route E, Figure 3) [25], but monomers arising from aryl-Cα cleavage and Cα-Cβ cleavage are also formed [24]. In model studies, oxidative cleavage by Dyp-type peroxidases has only been observed using units containing a free phenolic 4-hydroxyl group [6]; therefore, it seems likely that for breakdown of polymeric lignin breakdown, they cleave from the ends of a lignin chain (exo-cleavage), rather than in the middle of a chain (endo-cleavage). Expression of *R. jostii* DypB in tobacco plants has been shown to yield 200% more fermentable sugars, and reduced lignin content, demonstrating that Dyp-type peroxidases can be expressed heterologously to depolymerise lignin [39].

The reaction of multi-copper oxidases (laccases) with lignin often results in repolymerisation via phenoxy radical formation (route D, Figure 3) [18–21]. However, in the presence of mediators such as 1-hydroxybenzotriazole (HBT) or methyl syringate, detailed NMR studies have shown that oxidation of the α-hydroxyl group of the β-aryl ether unit (route A, Figure 3) is a major reaction [40,41]; however, studies of product release from lignin by fungal laccases have shown differences in behaviour depending on the mediator used [42]. Recent studies on *Amycolatopsis* SLAC have shown the release of products arising from aryl-Cα cleavage and Cα-Cβ cleavage [19].

**Figure 4**




Structures of engineered DyP-type peroxidases and location of residues which enhance their activity. a. Residues (L169 and L193) identified in *Pseudomonas fluorescens* Dyp1B using focused libraries. b. Residues (Y125, V142 and K188) found in *Pseudomonas putida* DyP using error-prone polymerase chain reaction. Models generated using Swiss-model (https://swissmodel.expasy.org/).

Beta-etherase enzymes which catalyse reductive ether cleavage (route F, Figure 3) had until recently only been shown to act on lignin dimer substrates [26–31] that may be lignin degradation intermediates. Combination of LigE and LigF with glutathione lyase LigG was found to be effective for bioconversion of lignin models [43]. However, there have been two significant recent reports of β-etherase enzymes acting on polymeric lignin substrates. Picart et al. have reported that treatment of beech wood lignin with laccase lcc2 M3 from *Trametes versicolor* in the presence of violuric acid, followed by β-etherases LigEG from *Sphingobium* SYK-6 and LigF from *Novosphingobium aromaticivorans*, yields a bio-oil containing low molecular weight aromatic compounds [44]. Furthermore, Gall et al. have shown that a combination of β-etherases LigDEFN and $NaGST_{NU}$, together with glutathione reductase to recycle reduced glutathione, releases guiaiacyl (G), syringyl (S), and tricin units from lignin oligomers and polymeric lignin [45]. These reports raise the possibility that this class of enzymes could be used to attack polymeric lignin.

## Lignin degradation accessory enzymes

One limitation of using recombinant lignin-oxidising enzymes for *in vitro* biotransformation of lignin substrates is that dimerization or repolymerisation is often observed, due to the formation of phenoxy radicals that spontaneously recombine. Therefore, it is likely that there are accessory enzymes *in vivo* that can trap phenoxy radicals via one-electron reduction. One candidate enzyme for this activity has been recently identified, a highly expressed extracellular dihydrolipoamide dehydrogenase from *T. fusca*, which has been shown to prevent dimerization of a lignin model compound *in vitro*, and change the profile of low molecular weight products formed [46]. Reductase enzymes such as this could be valuable accessory enzymes for lignin biotransformation.

Another accessory enzyme activity needed for lignin degradation *in vivo* is the generation of hydrogen peroxide co-substrate for lignin-oxidising peroxidase enzymes, probably generated from dioxygen by oxidase enzymes. Two such oxidase enzymes have been identified recently that appear to be linked to lignin degradation. A copper-dependent oxidase enzyme has been identified in *T. fusca*, which has been shown to result in reduced lignin content in sugarcane bagasse, and generates dilignol products [47]. A new pathway for metabolism of aryl-$C_2$ lignin fragments in *R. jostii* RHA1 has also been shown to involve an FMN-dependent oxidase enzyme that can oxidise aldehyde intermediates and simultaneously generate hydrogen peroxide [48].

## Protein engineering studies

Two recent reports describe the application of directed evolution methods to bacterial Dyp-type peroxidase enzymes. Brissos et al. report the engineering of *P. putida* DyP using error-prone polymerase chain reaction, giving a mutant enzyme containing three mutations (E188K, A142V, H12V), each on the surface of the enzyme (see Figure 4), that enhance $k_{cat}/K_M$ for 2,6-dimethoxyphenol by 100-fold, and shift the optimum pH to 8.5 [49]. Rahmanpour et al. report the use of focused libraries around the active site of *P. fluorescens* Dyp1B, enhancing the $k_{cat}/K_M$ for 2,4-dichlorophenol by 7–8 fold, and mutation H169L was found to enhance product release from polymeric lignin [50].

In summary, the study of microbial lignin degradation is leading to the discovery of a range of new enzyme biocatalysts that could be applied either *in vitro* or in whole

cell biotransformations for conversion of lignin to high-value chemicals.

## Conflict of interest statement

Nothing declared.

## Acknowledgements

Research in the author's laboratory is supported by research grants from BBSRC (research grant BB/P01738X/1) and the European Union Horizon 2020 research and innovation programme (Bio-Based Industries Joint Undertaking, grant agreement 720303).

## References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Fiorentino G, Ripa M, Ulgiati S: **Chemicals from biomass: technological *versus* environmental feasibility – a review**. *Biofuel Bioprod Bior* 2017, **11**:195–214.

2. Bugg TDH, Rahmanpour R: **Enzymatic conversion of lignin into renewable chemicals**. *Curr Opin Chem Biol* 2015, **29**: 10–17.

3. Gupta VK, Kubicek CP, Berrin JG, Wilson DW, Couturier M, Berlin A, Filho EXF, Ezeji T: **Fungal enzymes for bio-products from sustainable and waste biomass**. *Trends Biochem Sci* 2016, **41**:633–645.

4. Ahmad M, Taylor CR, Pink D, Burton K, Eastwood D, Bending GD, Bugg TDH: **Development of novel assays for lignin degradation: comparative analysis of bacterial and fungal lignin degraders**. *Mol Biosyst* 2010, **6**:815–821.

5. Taylor CR, Hardiman EM, Ahmad M, Sainsbury PD, Norris PR, Bugg TDH: **Isolation of bacterial strains able to metabolise lignin from screening of environmental samples**. *J Appl Microbiol* 2012, **113**:521–530.

6. Ahmad M, Roberts JN, Hardiman EM, Singh R, Eltis LD, Bugg TDH: **Identification of DypB from *Rhodococcus jostii* RHA1 as a lignin peroxidase**. *Biochemistry* 2011, **50**: 5096–5107.

7. Yoshida T, Sugano Y: **A structural and functional perspective of DyP-type peroxidase family**. *Arch Biochem Biophys* 2015, **574**:49–55.

8. Brown ME, Barros T, Chang MCY: **Identification and characterization of a multifunctional dye peroxidase from a lignin-reactive bacterium**. *ACS Chem Biol* 2012, **7**:2074–2081.

9. Rahmanpour R, Bugg TDH: **Characterisation of Dyp-type peroxidases from *Pseudomonas fluorescens* Pf-5: oxidation of Mn(II) and polymeric lignin by Dyp1B**. *Arch Biochem Biophys* 2015, **574**:93–98.

10. Rahmanpour R, Rea D, Jamshidi S, Fülöp V, Bugg TDH: **Structure of *Thermobifida fusca* DyP-type peroxidase and activity towards Kraft lignin and lignin model compounds**. *Arch Biochem Biophys* 2016, **594**:54–60.

11. Salvachua D, Karp EM, Nimlos CT, Vardon DR, Beckham GT: **Towards lignin consolidated bioprocessing: simultaneous lignin depolymerization and product generation by bacteria**. *Green Chem* 2015, **17**:4951–4967.

12. Salvachua D, Johnson CW, Singer CA, Rohrer H, Peterson DJ, Black BA, Knapp A, Beckham GT: **Bioprocess development for muconic acid production from aromatic compounds and lignin**. *Green Chem* 2018, **20**:5007–5019.

13. Lin L, Wang X, Cao L, Xu M: **Lignin catabolic pathways reveal unique characteristics of dye-decolorizing peroxidases in *Pseudomonas putida***. *Environ Microbiol* 2019, **21**:1847–1863.

14. Yang C, Yue F, Cui Y, Xu Y, Shan Y, Liu B, Zhou Y, Lu X: **Biodegradation of lignin by *Pseudomonas* sp. Q18 and the characterization of a novel bacterial DyP-type peroxidase**. *J Ind Microbiol Biotechnol* 2018, **45**:913–927.

15. Yu W, Liu W, Huang H, Zheng F, Wang X, Wu Y, Li K, Xie X, Jin Y: **Application of a novel alkali-tolerant thermostable DyP-Type peroxidase from *Saccharomonospora viridis* DSM 43017 in biobleaching of eucalyptus Kraft pulp**. *PLoS One* 2014, **9**. e110319.

16. Sahinkaya M, Colak DN, Ozer A, Canakci S, Deniz I, Belduz AO: **Cloning, characterization and paper pulp applications of a newly isolated DyP type peroxidase from *Rhodococc*ussp. T1**. *Mol Biol Rep* 2019, **46**:569–580.

17. * Shrestha R, Huang G, Meekins DA, Geisbrecht BV, Li P: **Mechanistic insights into dye-decolorizing peroxidase revealed by solvent isotope and viscosity effects**. *ACS Catal* 2017, **7**: 6352–6364.
This paper provides a more detailed mechanistic insight into the catalytic cycle of a bacterial dye-decolorizing peroxidase enzyme.

18. Majumdar S, Lukk T, Solbiati JO, Bauer S, Nair SK, Cronan JE, Gerlt JA: **Roles of small laccases from *Streptomyces* in lignin degradation**. *Biochemistry* 2014, **53**:4047–4058.

19. * Singh R, Hu J, Regner MR, Round JW, Ralph J, Saddler JN, Eltis LD: **Enhanced delignification of steam-pretreated poplar by a bacterial laccase**. *Sci Rep* 2017, **7**:42121.
This study demonstrates that bacterial multi-copper oxidase enzymes can be used to generate enhanced yields of acid-precipitable lignin and specific monocyclic aromatic products from lignin breakdown.

20. Granja-Travez RS, Wilkinson RC, Persinoti GF, Squina FM, Fülöp V, Bugg TDH: **Structural and functional characterisation of a multi-copper oxidase CueO from lignin-degrading bacterium *Ochrobactrum* sp. reveal its activity towards lignin model compounds and lignosulfonate**. *FEBS J* 2018, **285**: 1684–1700.

21. Granja-Travez RS, Bugg TDH: **Characterisation of multi-copper oxidase CopA from *Pseudomonas putida* KT2440 and *Pseudomonas fluorescens* Pf-5: involvement in bacterial lignin oxidation**. *Arch Biochem Biophys* 2018, **660**: 97–107.

22. Soldatova AV, Tao L, Romano CA, Stich TA, Casey WH, Britt RD, Tebo BM, Spiro TG: **Mn(II) oxidation by the multicopper oxidase complex Mnx: a binuclear activation mechanism**. *J Am Chem Soc* 2017, **139**:11369–11380.

23. Mathews SL, Smithson CE, Grunden AM: **Purification and characterization of a recombinant laccase-like multi-copper oxidase from *Paenibacillus glucanolyticus* SLM1**. *J Appl Microbiol* 2016, **121**:1335–1345.

24. Rashid GMM, Taylor CR, Liu Y, Zhang X, Rea D, Fülöp V, Bugg TDH: **Identification of manganese superoxide dismutase from *Sphingobacterium sp.* T2 as a novel bacterial enzyme for lignin oxidation**. *ACS Chem Biol* 2015, **10**: 2286–2294.

25. * Rashid GMM, Zhang X, Wilkinson RC, Fülöp V, Cottyn B, Baumberger S, Bugg TDH: ***Sphingobacterium* sp. T2 manganese superoxide dismutase catalyses the oxidative demethylation of polymeric lignin via generation of hydroxyl radical**. *ACS Chem Biol* 2018, **13**:2920–2929.
This paper elucidates a novel mechanism of action of an unusual manganese superoxide dismutase enzyme that can generate hydroxyl radical to attack lignin, and explores the molecular basis for this unusual reactivity.

26. Masai E, Ichimura A, Sato Y, Miyauchi K, Katayama Y, Fukuda M: **Roles of the enantioselective glutathione *S*-transferases in cleavage of β-aryl ether**. *J Bacteriol* 2003, **185**:1768–1775.

27. Tanamura K, Abe T, Kamimura N, Kasai D, Hishiyama S, Otsuka Y, Nakamura M, Kajita S, Katayama Y, Fukuda M, Masai E: **Characterization of the third glutathione S-transferase gene involved in enantioselective cleavage of the β-aryl ether by *Sphingobium* sp. strain SYK-6**. *Biosci Biotechnol Biochem* 2011, **75**:2404–2407.

28. Gall DL, Kim H, Lu F, Donohue TJ, Noguera DR, Ralph J: **Stereochemical features of glutathione-dependent enzymes in the *Sphingobium* sp. strain SYK-6 β-aryl etherase pathway**. *J Biol Chem* 2014, **289**:8656–8667.

29. Pereira JH, Heins RA, Gall DL, McAndrew RP, Deng K, Holland KC, Donohue TJ, Noguera DR, Simmons BA, Sale KL, Ralph J, Adams PD: **Structural and biochemical characterization of the early and late enzymes in the lignin β-aryl ether cleavage pathway from *Sphingobium* sp. SYK-6**. *J Biol Chem* 2016, **291**:10228–10238.

30. Ohta Y, Nishi S, Hasegawa R, Hatada Y: **Combination of six enzymes of a marine *Novosphingobium* converts the stereoisomers of β-*O*-4 lignin model dimers into the respective monomers**. *Sci Rep* 2015, **5**. article 15105.

31. Kontur WS, Bingman CA, Olmsted CN, Wassarman DR, Ulbrich A, Gall DL, Smith RW, Yusko LM, Fox BG, Noguera DR, Coon JJ, Donohue TJ: ***Novosphingobium aromaticivorans* uses a Nu-class glutathione *S*-transferase as a glutathione lyase in breaking the β-aryl ether bond of lignin**. *J Biol Chem* 2018, **293**:4955–4968.

32. Marinović M, Nousiainen P, Dilokpimol A, Kontro J, Moore R, Sipilä J, de Vries RP, Mäkelä MR, Hildén K: **Selective cleavage of lignin β-O-4 aryl ether bond by β-etherase of the white-rot fungus *Dichomitus squalens***. *ACS Sustainable Chem Eng* 2018, **6**:2878–2882.

33. Ho JCH, Pawar SV, Hallam SJ, Yadav VG: **An improved whole-cell biosensor for the discovery of lignin-transforming enzymes in functional metagenomic screens**. *ACS Synth Biol* 2018, **7**:392–398.

34. Sana B, Burton Chia KH, Raghavan SS, Ramalingam B, Nagarajan N, Seayad J, Ghadessy FJ: **Development of a genetically programmed vanillin-sensing bacterium for high-throughput screening of lignin-degrading enzyme libraries**. *Biotechnol Biofuels* 2017, **10**. article 32.

35. Jha RK, Bingen JM, Johnson CW, Kern TL, Khanna P, Trettel DS, Strauss CEM, Beckham GT, Dale T: **A protocatechuate biosensor for *Pseudomonas putida* KT2440 via promoter and protein evolution**. *Metab Eng Commun* 2018, **6**:33–38.

36. Machado LFM, Dixon N: **Development and substrate specificity screening of an *in vivo* biosensor for the detection of biomass derived aromatic chemical building blocks**. *Chem Commun* 2016, **52**:11402–11405.

37. Moraes EC, Alvarez TM, Persinoti GF, Tomazetto G, Brenelli LB, Paixão DAA, Ematsu GC, Aricetti JA, Caldana C, Dixon N, Bugg TDH, Squina FM: **Lignolytic-consortium omics analyses reveal novel genomes and pathways involved in lignin modification and valorization**. *Biotechnol Biofuels* 2018, **11**. article 75.

38. Lancefield CS, Rashid GMM, Bouxin F, Wasak A, Tu W-C, Hallett J, Zein S, Rodríguez J, Jackson SD, Westwood NJ, Bugg TDH: **An investigation of the chemocatalytic and biocatalytic valorisation of a range of different lignin preparations: the importance of β-O-4 content**. *ACS Sustainable Chem Eng* 2016, **4**:6921–6930.

39. Ligaba-Osena A, Hankoua B, DiMarco K, Pace R, Crocker M, McAtee J, Nagachar N, Tien M, Richard TL: **Reducing biomass recalcitrance by heterologous expression of a bacterial peroxidase in tobacco (*Nicotiana benthamiana*)**. *Sci Rep* 2017, **7**:17104.

40. Crestini C, Jurasek L, Argyropoulos DS: **On the mechanism of the laccase–mediator system in the oxidation of lignin**. *Chem Eur J* 2003, **9**:5371–5378.

41. Rico A, Rencoret J, del Rio JC, Martinez AT, Gutierrez A: **In-depth 2D NMR study of lignin modification during pretreatment of *Eucalyptus* wood with laccase and mediators**. *Bioenergy Res* 2015, **8**:211–230.

42. Longe LF, Couvreur J, Leriche Grandchamp M, Garnier G, Allais F, Saito K: **Importance of mediators for lignin degradation by fungal laccase**. *ACS Sustainable Chem Eng* 2018, **6**: 10097–10107.

43. Picart P, Sevenich M, Domínguez de María P, Schallmey A: **Exploring glutathione lyases as biocatalysts: paving the way for enzymatic lignin depolymerization and future stereoselective applications**. *Green Chem* 2015, **17**: 4931–4940.

44. ** Picart P, Liu H, Grande PM, Anders N, Zhu L, Klankermayer J, Leitner W, Domínguez de María P, Schwaneberg U, Schallmey A: **Multi-step biocatalytic depolymerization of lignin**. *Appl Microbiol Biotechnol* 2017, **101**:6277–6287.

This study combines a laccase and β-etherase treatment of polymeric lignin, showing that a combination of two enzymes acting on lignin can be more effective than one.

45. ** Gall DL, Kontur WS, Lan W, Kim H, Li Y, Ralph J, Donohue TJ, Noguera DR: ***In vitro* enzymatic depolymerization of lignin with release of syringyl, guaiacyl, and tricin units**. *Appl Environ Microbiol* 2018, **84**. e02076-17.

This study demonstrates that β-etherase enzymes can be active on polymeric lignin in an *in vitro* biotransformation.

46. Rahmanpour R, King LDW, Bugg TDH: **Identification of an extracellular bacterial flavoenzyme that can prevent re-polymerisation of lignin fragments**. *Biochem Biophys Res Commun* 2017, **482**:57–61.

47. Lee CC, Chen HS, Yang CH, Wang SP, Wu JH, Meng M: **Modification of lignin in sugarcane bagasse by a mono-copper hydrogen peroxide-generating oxidase from *Thermobifida fusca***. *Process Biochem* 2016, **51**:1486–1495.

48. Wei Z, Wilkinson RC, Rashid GMM, Brown D, Fülöp V, Bugg TDH: **Characterization of thiamine diphosphate-dependent 4-hydroxybenzoylformate decarboxylase enzymes from *Rhodococcus jostii* RHA1 and *Pseudomonas fluorescens* Pf-5 involved in degradation of aryl $C_2$ lignin degradation fragments**. *Biochemistry* 2019, **58**:5281–5293.

49. Brissos V, Tavares D, Sousa AC, Robalo MP, Martins LO: **Engineering a bacterial DyP-type peroxidase for enhanced oxidation of lignin-related phenolics at alkaline pH**. *ACS Catal* 2017, **7**:3454–3465.

50. Rahmanpour R, Ehibhatiomhan A, Huang Y, Ashley B, Rashid GMM, Mendel-Williams S, Bugg TDH: **Protein engineering of *Pseudomonas fluorescens* peroxidase Dyp1B for oxidation of phenolic and polymeric lignin substrates**. *Enzym Microb Technol* 2019, **123**:21–29.